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PRINCIPAL INVESTIGATOR: Jason A. Koutcher, M.D., Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Cancer Center
New York, New York 10021-6007

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13. ABSTRACT *(Maximum 200 words)*

Previous studies in murine tumors have shown that the 3 drug combination of PALA + MMPR + 6AN [PALA (n-phosphonacetyl aspartate), MMPR (6-methylmercaptopurine riboside), 6AN (6-aminonicotinamide); referred to as PMA] is an effective radiation sensitizer. Using the MCF-7 human tumor, we have studied the effects of these drugs on metabolism and cell survival. In vivo studies demonstrate the presence of 6-phosphogluconate (6PG) and a reduction in nucleoside triphosphates after treatment with PMA. MCF-7 tumors were found to be more sensitive to radiation than previously studied murine tumors. PMA is more toxic to nude mice than to C3H mice but appears to be tolerable, although further studies are ongoing. In vitro studies with MCF-7 cells demonstrated that 6AN is not toxic by itself, but does induce metabolic inhibition, based on depletion of NTP and the appearance of 6PG. Ongoing in vivo studies include completion of the 31P NMR metabolic studies and tumor growth delay assays. In vitro studies include cell survival studies on the effect of 6AN in enhancing radiation response.

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Introduction

Previous studies by this investigator have shown that PMA[PALA + MMPR + 6AN [PALA (n-phosphonacetyl aspartate), MMPR (6-methylmercaptopurine riboside), 6AN (6-aminonicotinamide); referred to as PMA] is an effective radiosensitizer and enhances chemotherapy in two tumor models (1-5). In addition, we have shown that 6AN enhances the effect of radiation on RIF-1 fibrosarcoma cells (6,7). This enhancement has been shown to be sequence dependent, i.e. if the radiation is administered prior to 6AN there is no enhancement. A criticism of the study that has been done was that the study was done in murine tumors; the NIH has suggested that human tumors need to be studied. Thus the goal of the current studies are to

1. Determine if 6AN enhances the effects of radiation in cell culture with a human tumor model.
2. Reproduce our previous murine tumor data (NMR (nuclear magnetic resonance) and tumor growth delay studies) in a human tumor (in vivo).

Body

During the previous funding interval, the following studies have been completed or are progressing.

Studies were begun with the MCF-7 tumor model, both in vivo and in vitro. In vivo, we have studied 4 mice treated with PALA + MMPR + 6AN [PALA (n-phosphonacetyl aspartate), MMPR (6-methylmercaptopurine riboside), 6AN (6-aminonicotinamide); referred to as PMA] at the same doses used for studying murine tumors (PALA-100mg/kg, MMPR – 150mg/kg, and 6AN (10mg/kg) and followed by NMR. The mice were studied prior to treatment, and at 3, 10 and 24 and 48 hours post treatment. Tumors were transplanted in the mammary fat pad (orthotopic site) as recommended by the site visit team. There were some initial problems in obtaining tumor growth at the orthotopic site but these were resolved. The tumors typically required approximately 6 weeks to grow to a volume of approximately 300 mm3, the minimum size that is feasible for 31P NMR studies. The mice were also implanted with a time release estrogen pellet since the MCF-7 is estrogen dependent. Chemotherapy and radiation were administered as previously published (1-4).

A typical series of studies is shown in Fig. 1. As was noted for the two murine tumors studied previously, PMA causes an increase in 6PG (otherwise undetectable) and a decrease in high energy phosphates. These effects have not been quantitated yet since the studies are ongoing. These studies are more complex than previous studies since the tumors are implanted in the mammary fat pad and therefore the spectra require localization to avoid obtaining signal from the adjacent normal tissue. A one dimensinal chemical shift imaging technique we have used previously (8) is employed to selectively obtain signal from the tumor. The mice are imaged initally, to ascertain precisely where the tumor is located, and the coil parameters determined by optimizing the signal on a phantom of methylene diphosphonate, and subsequently spectral data obtained. After completion of the study, the data are transferred to a SUN workstation, where the images are displayed and the data "voxel shifted" so that signal is selected from the tumor and not adjacent muscle as described previously (8). The data are then reconstructed and metabolite intensities measured. Studies on 4 mice have been completed and we intend to study an additional 4 mice to complete this aspect. The use of a different site of tumor implantation did require some modifications to the radiofrequency coil and data acquisition parameters, but these have been resolved.

We began studies to monitor the radiation enhancement of this regimen. The first cohort of mice treated with radiation alone (15 Gy fraction x 3 fractions separated by 10 or 11 days) demonstrated that this tumor was far more radiation sensitive than the two murine tumors studied. The tumors shrunk after the first dose of radiation. The starting tumor volume was 137.0 $\pm$ 5.5 mm3 (mean $\pm$ SEM). By day 61, the tumor had shrunk to 54.1 $\pm$ 4.3 mm3. In comparison, previous studies with the CD8F1 tumor with this fractionation schedule had shown that almost all tumors had doubled by day 40. Since this tumor is far more sensitive than expected and therefore enhancement would be difficult to demonstrate with this fractionation schedule, a second cohort was studied receiving 10

Gy fractions instead. These results showed that the tumor grew from a mean volume of 138.9 $\pm$ 3.7 at the start of treatment to a volume of 104.1 $\pm$ 25 on day 40). This response suggests that a 10 Gy fraction will be appropriate for the combined PMA + XRT study.

Simultaneously, we began treating mice with PMA, to ensure that the mice would tolerate this dose of chemotherapy. Surprisingly, the first cohort demonstrated a 4/5 mortality rate. This high mortality was thought to be due to problems with temperature control in the room and also dehydration. A second cohort was treated with the same dose with 6/6 surviving, although one mouse was sacrificed because of weight loss. A third cohort to confirm the safety of this regimen has just been started.

In the initial plans for the study, we had hoped to study two murine tumor models. The use of a human tumor, implanted at an orthotopic site, has led to some unexpected problems which have been resolved. The delays caused by the use of a model which was new to us has led us to focus on the single tumor model to ensure that the experiments were done correctly. It is unclear whether during this time period we will be able to pursue and complete studies on the second tumor model (estrogen independent MDA-MB-468).

In addition, in vitro studies have been done with 6AN using the MCF-7. These studies utilize previously published techniques used by these investigators (6,7). Fig. 2 shows a series of spectra on MCF-7 cells obtained without treatment. These demonstrate that the cells are stable in the NMR perfusion system for extended periods of time. In contrast, Fig. 3 shows a series of spectra obtained on MCF-7 cells treated with 6AN. The results are similar to the RIF-1 results except that there is a decrease in NTP. Previous studies with RIF-1 used both a 15 hour exposure (6) and a 4 hour exposure (7). The 4 hour study was deemed superior since shorter infusions would be more relevant clinically.

Surviving fraction studies were done on the MCF-7 cells using a 4 hour exposure using previously described techniques (6,7). 6AN by itself, over the dose range of 40-240 uM showed no effect on survival as expected (Fig. 4). This is similar to our results with RIF-1 cells wherein 6AN alone had no effect on surviving fraction. Longer incubations did show that 6AN was slightly toxic at the higher doses. Further studies are ongoing to determine surviving fraction with radiation (3 and 6 Gy doses) in the presence and absence of 6AN.

Legends

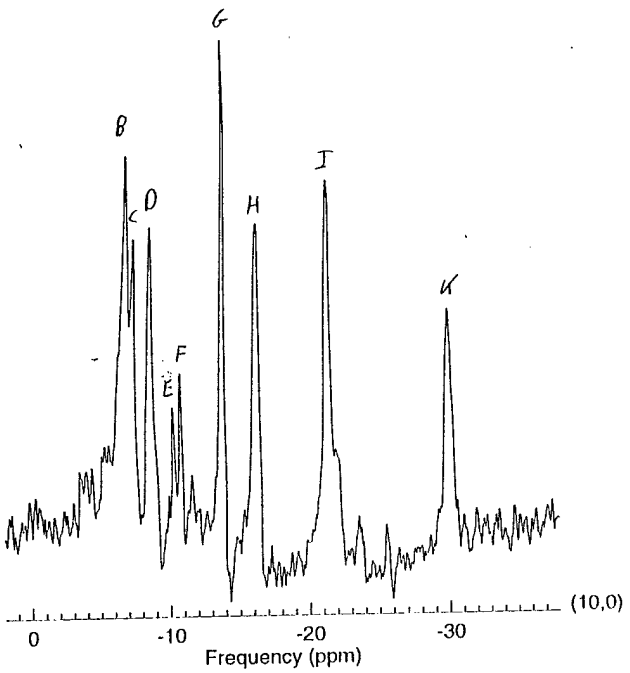
Fig. 1. In vivo 31P NMR studies of MCF-7 tumors treated with PMA. Peak identifications include A=6-phosphogluconate, B= phosphoethanolamine, C=phosphocholine, D=inorganic phosphate, E=glycerophosphoethanolamine, F=glycerophosphocholine, G=phosphocreatine, H=γ nucleoside triphosphate (NTP), I= α NTP, K=βNTP. Note the appearance of 6PG at 10 and 24 hours and the increase in Pi relative to β NTP.

Fig. 2. In vitro 31P NMR spectra of MCF-7 cells. Peak identification as in Fig. 1. The cells are metabolically stable over the duration of study. The difference between the top 4 spectra and 2 bottom spectra are caused by a change in the acquisition parameters (block size).

Fig. 3. In vitro 31P NMR spectra of MCF-7 cells perfused with 6AN for 4 hours. Note the appearance of 6PG and the decrease in phosphocreatine. A. Before 6AN. B. Post 6AN infusion. Each spectrum required 4.5 hours. Time points are from 6AN infusion.

Fig. 4. Surviving fraction studies done on MCF-7 cells at various doses of 6AN for various exposure durations. Note the lack of toxicity of 6AN at 200 uM when exposure is 4 hours.

BASELINE



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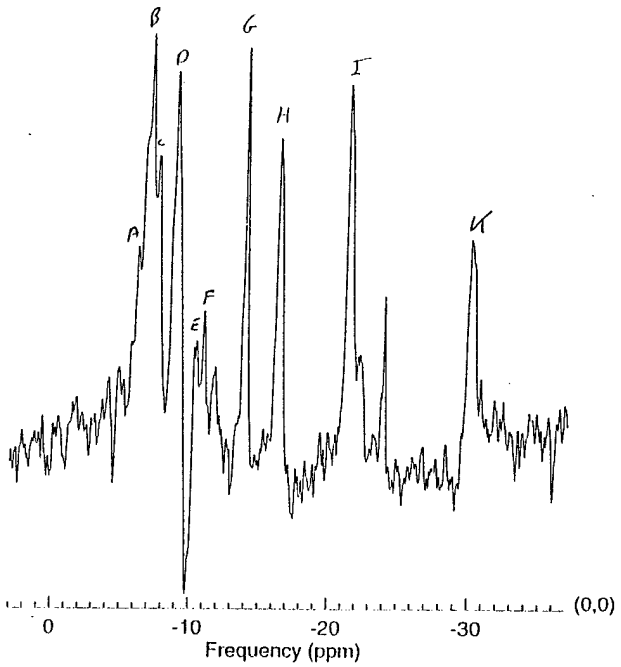


24 hours

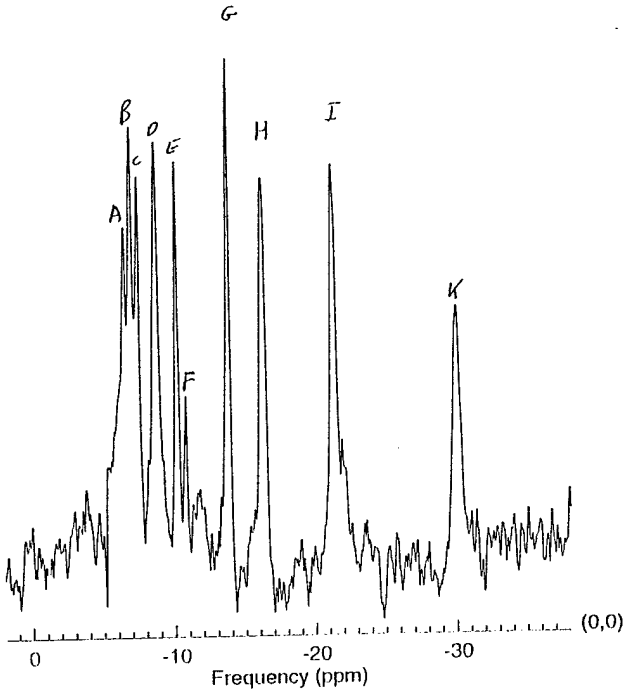
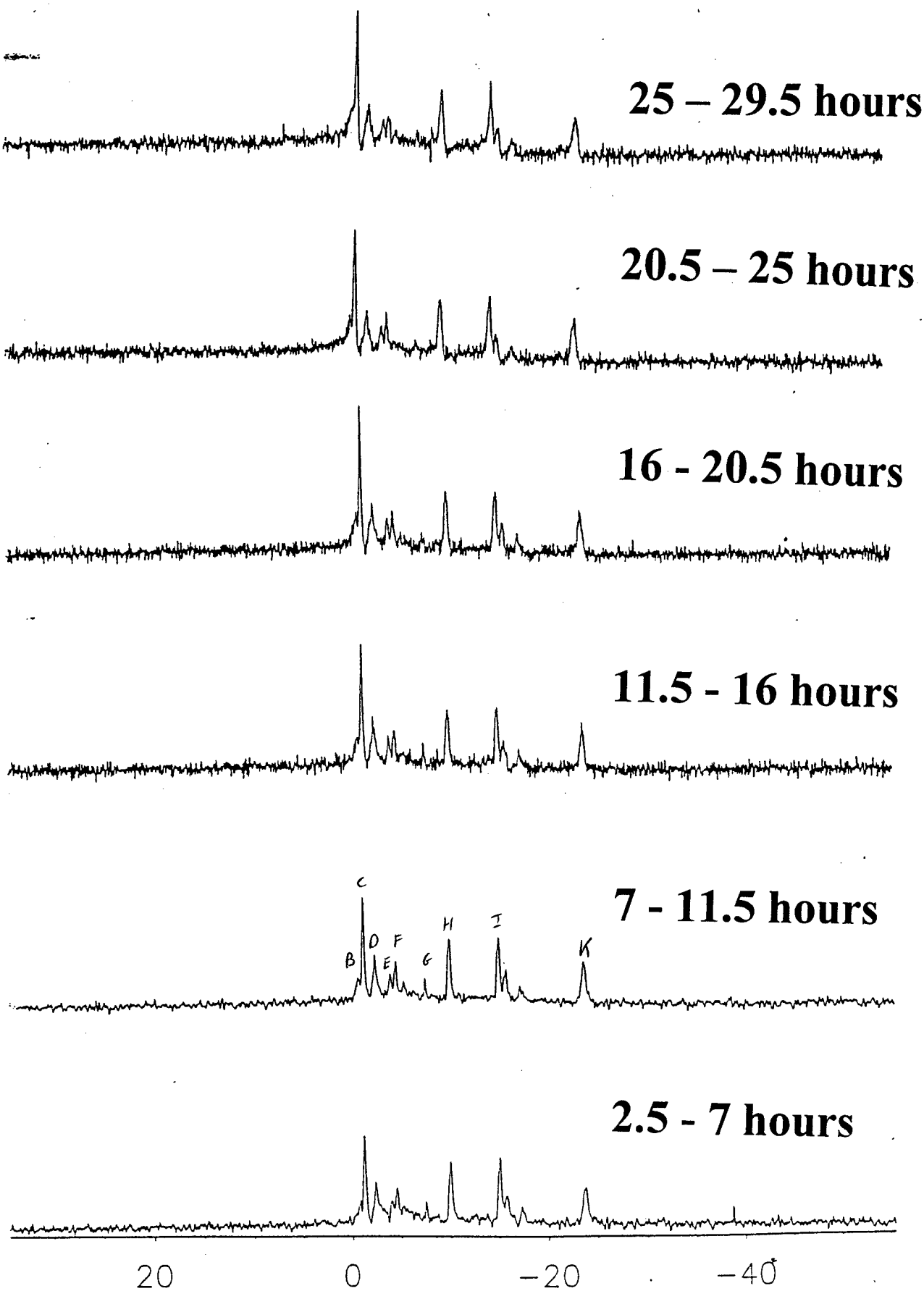


Figure 1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20.5 – 25 hours
16 - 20.5 hours
11.5 - 16 hours
7 - 11.5 hours
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2.5 - 7 hours
20
0
−20
−40
ppm

Figure 2

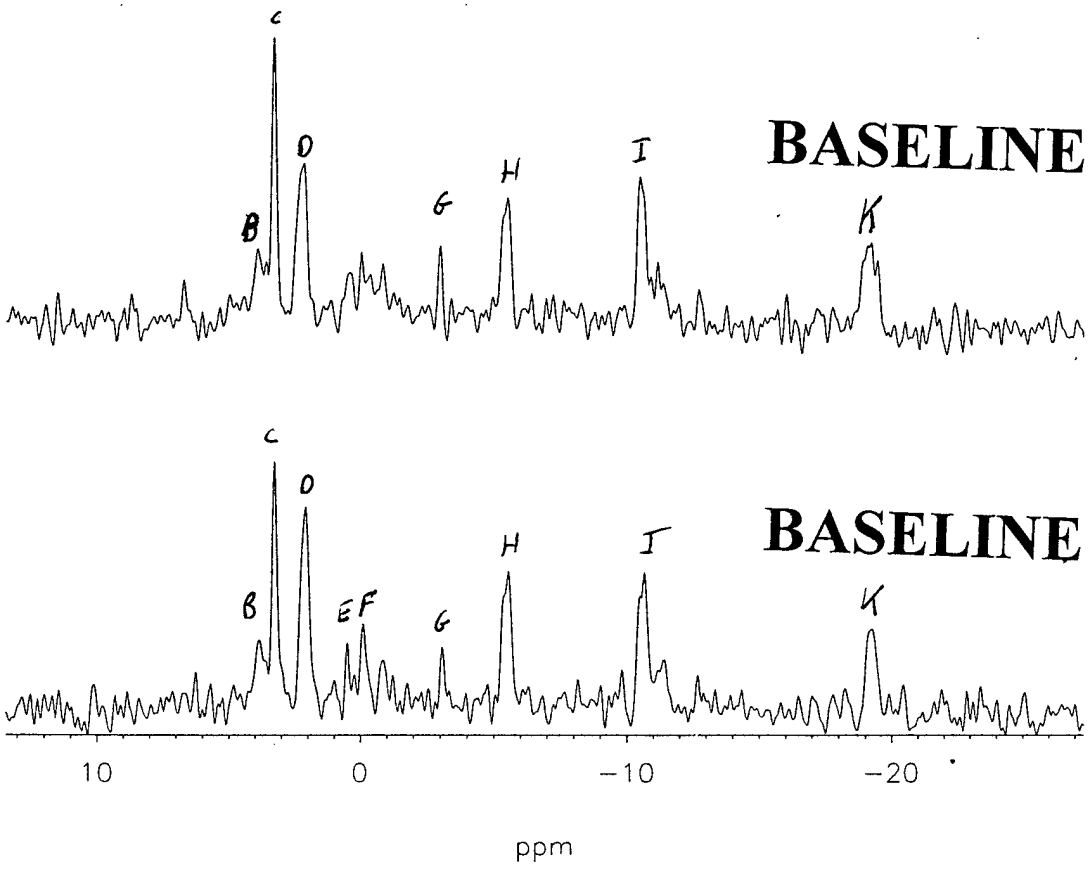

Figure 3A

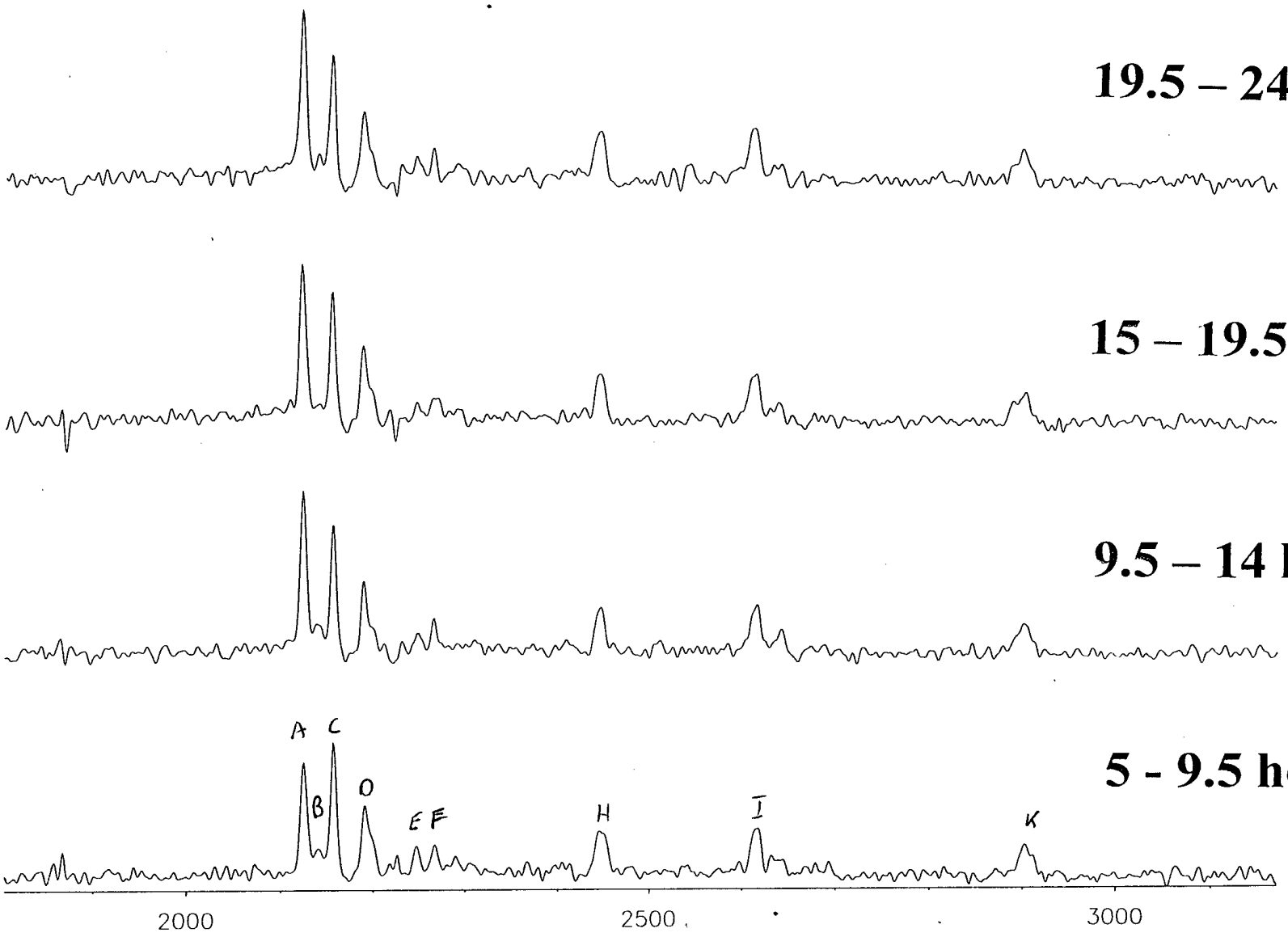
19.5 – 24 hours
15 – 19.5 hours
9.5 – 14 hours
5 - 9.5 hours
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Figure 3B

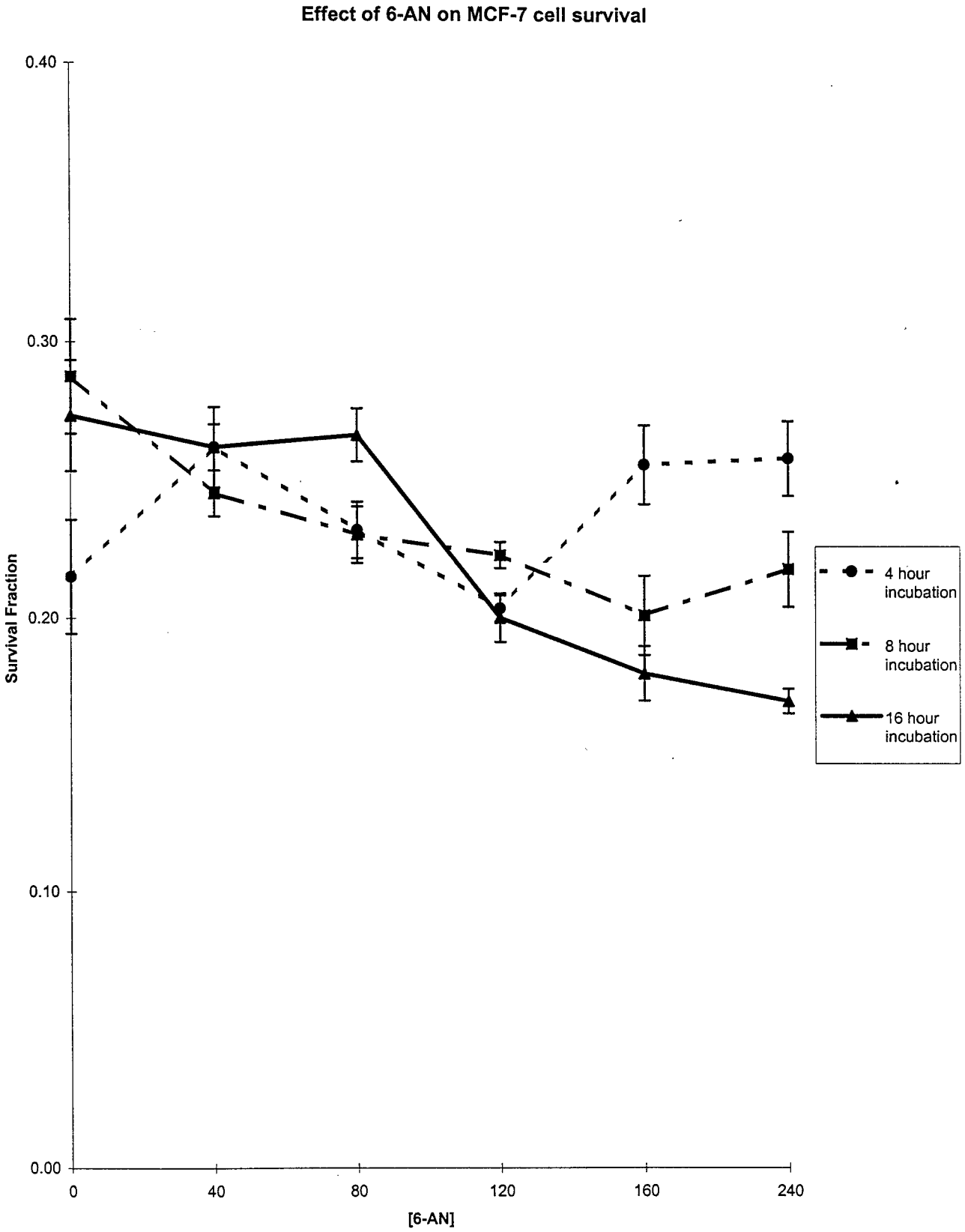
Effect of 6-AN on MCF-7 cell survival
0.40
0.30
0.20
0.10
0.00
Survival Fraction
0
40
80
120
160
240
[6-AN]
4 hour incubation
8 hour incubation
16 hour incubation

Figure 4

Conclusions

1. In vitro, 6AN is not toxic to human MCF-7 breast cancer cells in doses ranging from 40um to 240 uM at 4 hour exposures.
2. MCF-7 tumors (in vivo) are more radiation sensitive than two murine breast tumor models studied previously.
3. Nude mice are more sensitive to the PMA combination and need fluid supplementation and careful temperature monitoring.
4. Metabolic response to PMA in the MCF-7 showed 6-phosphogluconate and some decrease in NTP. The latter effect may be diminished in comparison to murine tumors. These studies are ongoing.

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